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Research Article

COMPARATIVE OSSIFICATION OF THE SKULL IN THREE NIGERIAN BREEDS OF SHEEP: AN ALIZARIN TECHNIQUE

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ABSTRACT: This study describes the ossification time, sequence, and morphogenic pattern of the neuro-cranial and viscera-cranial bones of the skull in three Nigerian breeds of sheep. A total number of four hundred and ninety (490) wasted fetuses were collected from abattoirs. The heads of the fetuses were severed and processed using the Alizarin technique. The ossifying bones were identified, and their morphology was mapped using FastStone Image software. In the three breeds of sheep, the neuro-cranial and viscera-cranial bones developed via intra-membranous ossification. Generally, bony spicules appeared to radiate from the ossification centers of the skull bones faster in Yankasa than Balami and Uda. The frontal, parietal, and the zygomatic process of frontal bones were the first of the neuro-cranial bones to begin ossification. The premaxilla, zygomatic, and maxilla were the first of the viscera-cranial bones to begin ossification. The earliest morphogenic pattern of the neuro-cranium, the occipital condyle was comma-shaped, the parietal and frontal were irregular, and the zygomatic process of temporal bones and frontal process of zygomatic were finger-like projections. Whereas among viscera-cranium, the premaxilla, nasal, and zygomatic were spindle-shaped, the maxilla and lacrimal were triangular and cube-shaped in all the three breeds of sheep. The ossification centers of the skull bones started from the calvarium and developed faster in Yankasa and the shape of the developing bones-varies as the bone spicules radiates and fetuses advanced with age. The temporal and interparietal ossification did not occur in the 1st and early 2nd trimester fetuses (42-67 days of gestation) of the three breeds of sheep. It was concluded that the frontal and premaxilla were the first bones of the skull to ossify and ossification of the viscera-cranium occurred earlier in Yankasa than in Balami and Uda and the ossification centers of all the neuro-cranial bones and nasal bones were eccentric.

Key words: Nigerian sheep, Viscero-cranium, Neuro-cranium, Fetus, Ossification, Skull.

INTRODUCTION

The bones which form the skeleton of the head (skull) have been divided into the viscerocranium and neurocranium, which forms the face and protective covering of the brain respectively. The neurocranium has two groups of bones; the basal bones and the calvarium (cranial vault) (Dyce *et al*. 2017). Understanding the congenital anomalies of the skull requires an understanding of the normal morphogenesis of the cranium (Lafci Fahrioglu *et al*. 2020). The bones of the skull are derived from the mesoderm and cranial neural crest (CNC). The cells of the CNC originate from the neural epithelial cells in the neural folds. These cells transform from epithelial to mesenchymal cells and migrate to their final destinations (ossification centers) in the craniofacial region where they condense to form membranous and cartilaginous tissues which later ossify to form the bones of the skull (Dolack *et al*. 2020). The study on the time of appearance of primary ossification centers of the skull is of great help in approximate age estimation during prenatal life, assessment of fetal bone maturation, and thus helps in the detection of some fetal abnormalities (Oishi *et al*. 1996). The study of normal embryonic and fetal growth can serve as a guide for understanding the consequences of harmful influences at various stages of gestation (Evans and Sack 1973). Several studies have reported the time and pattern of ossification in humans and mice (Marghoub *et al*. 2019, Susan 2015). However, there is a dearth of information in other species, especially in ruminants. Therefore, this

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study was carried out to establish the ossification time, morphology, sequence of appearance of the skull bones of fetuses of Nigerian breeds of sheep using the Alizarin staining technique.

MATERIALS AND METHODS

Samples collection and determination of age

A total of four hundred and ninety (490) wasted samples across three Nigerian breeds of sheep (Balami, Uda, and Yankasa) used for this study (Table 1) were procured from different abattoirs within Northern Nigeria. The fetal samples were brought to the department laboratory for further analysis. Their crown vertebral rump length (CVRL) were measured with a measuring tape, from the anterior fontanel, and following the vertebral column down to the base of the tail to determine the gestational ages in days as described by Arthur *et al*. (1989) using the formula: X = 2.1(Y+17) where X= Gestational age in days and Y= Crown vertebral rump length. The CVRL of the fetuses ranged from 3.0-15 cm (Table 1).

Preparation of fetal skull for the Alizarin staining technique

All the fetuses were subjected to the Alizarin staining technique; modified from Salaramoli *et al.* (2015) and Ahmed (2008) (Fig. 1).

The fetal specimens were completely eviscerated, rinsed in tap water, and immersed in a water bath at 70^{o}C for approximately 30 seconds before skinning with a scalpel blade. The samples were fixed in 95% ethanol (Loba Chemie®, Mumbai India) for 6-8 hours depending on the size of the fetus. The fixed fetuses were stained with a mixed alcoholic solution consisting of 0.3% Alizarin red (Loba Chemie®, Mumbai India) for 17 hours.

The bones were macerated using 2% potassium hydroxide (KOH) (Loba Chemie®, Mumbai India) for 8 hours. The maceration time and KOH concentration were varied according to the age of the fetus; KOH 2-10% concentration was used for about 6-24 hours, head of the older fetuses were macerated in a higher concentration of above 8% for 20-24 hours.

The macerated samples were cleared using gradual concentrations of glycerin (Loba Chemie®, Mumbai India) in distilled water (20%, 50%, and 80%) for 14 hours. The stained samples were examined in the glycerin-distilled water mixture using a dissecting microscope (Leica®) to detect the sequence of appearance of the primary ossification centers and pattern of ossification.

The stereo-micrograph of the stained samples was captured, and images uploaded on a personal computer and mapping of ossification centers and ossified parts were performed using Fast Stone Image software version 4.

Table 1. Age group classification for fetuses collected from the slaughter house.

Age group	CVRL (cm)	Balami (n)	Uda (n)	Yankasa (n)	Age (days)	Trimester
One	3.0-3.9	10	30	30	42-44	1st
Two	4.0-9.0	10	30	30	44-46	1st
Three	5.0-5.9	10	30	30	46-48	1st
Four	6.0-6.9	10	30	30	48-50	1st
Five	7.0-7.9	10	30	30	50-52	2nd
Six	8.0-8.9	10	30	30	53-54	2nd
Seven	9.0-15	10	30	30	55-67	2nd
Total		70	210	210		490

RESULTS AND DISCUSSION

Development of the neurocranial bones of the skull

The time, sequence, and structure of the following neurocranial bones during ossification were observed in the present study: occipital bone (OC), parietal bone (PA), temporal bone (TP), and frontal bone (FT), the zygomatic process of the frontal bone (ZPf), the zygomatic process of the temporal bone (ZPt). In the first trimester, the membranous tissues of the parietal (PA), frontal (FT), and the zygomatic process of the temporal (ZPt) bones began to ossify at 45th - 47th days of gestation in Yankasa and 48th – 50th days of gestation in Uda and Balami breeds of sheep (Fig. 2 and Fig. 3). The ossifying parietal and frontal bone appeared as a meshwork of bony spicules with a suture line separating the two bones. The ossification of the left and right halves of parietal and frontal bones both started from the left and right lateral parts and progressed towards the median plane with an irregular medial margin where both of them apposed (Fig. 3-5). In the first trimester, the zygomatic process of the temporal bone (ZPt) appeared as a finger-like projection extending caudally from the caudoventral part of the orbit above the temporal process of zygomatic bone (TPz) to the ventral part of the developing parietal bone spicules (Fig. 2 - 3). The developing zygomatic process of the temporal bone (ZPt) had a wide anastomosing bony spicule radiating from its caudal end. However, the ossification of the temporal bone (TP) did not occur in all the age groups (42nd – 67th days of gestation) (Fig. 2-8).

A small dense comma-shaped ossification centre of the occipital bone (OS) (squamous part) appeared at 51st - 53rd days of gestation in Yankasa and 54th - 56th days of

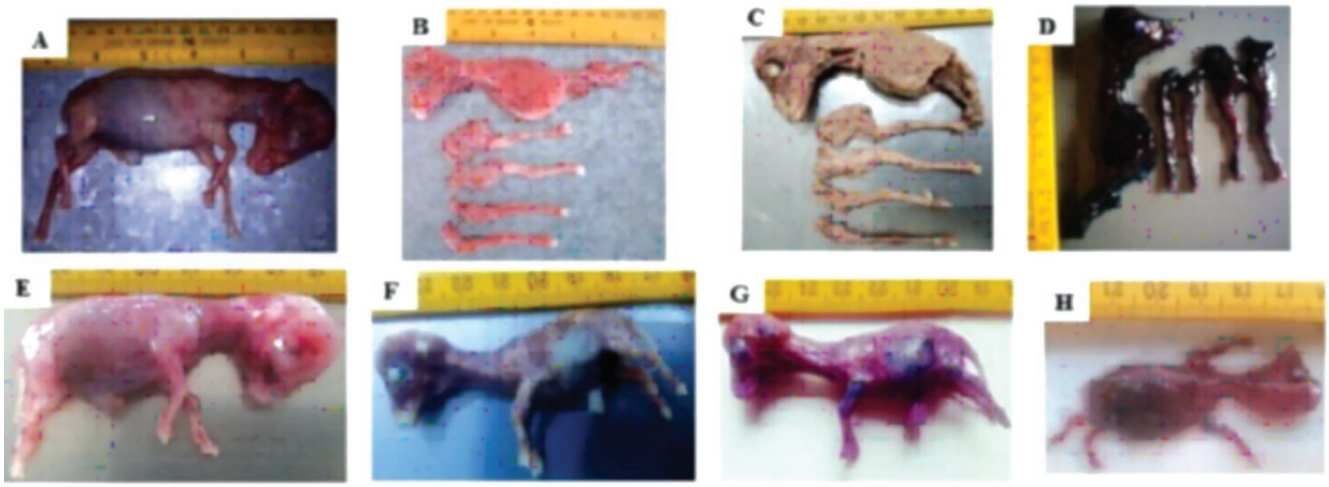

Fig. 1. Photographs of the stages of the single staining procedures; Fresh Uda fetal samples (A and E), Skinned and Eviscerated fetus (B), Ethanol Fixed fetuses (C and F), Alcian stained and KOH Treated fetuses (D and G) and Glycerine cleared fetus (H).

gestation in Uda and Balami (Fig. 5). With the age of the fetus, the ossification of the occipital squamous widened progressively towards the left and right lateral parts from the comma to a butterfly-shaped bone with an evident dorsal margin of foramen magnum at 61st day and later to a block arc-shape at 65th days of gestation (Fig. 6 – 7). However, at 48th - 49th days of gestation in Yankasa and 49th - 50th days of gestation in Balami and Uda, the paired bony spicules forming the ossification centers of the interparietal part of the occipital bone (IO) emerged caudal to the left and right ossifying parietal bone, and at 61st day of gestation, the paired bony spicules forming the IO united to form a single bone (Fig. 6). With the age of the fetus (65th day) the uniform spicules spread to form irregularly arranged spicules (Fig. 7).

A membrane bone is a bony structure derived from intra-membranous ossification which forms bones of the skull (de Buffrénil *et al*.2015). Understanding the development of a skull deformity requires an understanding of the normal ossification process of the cranium (Sung-Won Jin *et al.* 2016). The frontal bone (FT), parietal bone (PA), and the zygomatic process of the temporal bone (ZPt) began ossification earlier in Yankasa than in the Uda and Balami, with their eccentric ossification centers. The radiation of bone spicules from the caudal end of the ZPt indicates that the ossification centre of the temporal bone emerges from its zygomatic process. The ossification centers of these bones were situated on both lateral sides at a point where it developed into bony spicules that spread towards the middle in frontal and parietal bones while the bone spicules of the zygomatic process of temporal spread caudally, these signify eccentric ossification centers occur in the bones. The actual time of ossification of these bones is not properly reported in most species of animals. However, in humans, it was reported by Susan (2015) that the parietal bone began ossification at a later age (56th day of gestation). Although the parietal bone in this study arises from intra-membranous ossification as in humans, the location and number of ossification centers of this bone differ from that of humans. The pattern and sequence of ossification in the sheep do not differ essentially from that of humans. The bony spicules observed during the ossification of the frontal bone, parietal bone, and zygomatic process of the temporal bone in this study signifies that they all ossify via intra-membranous ossification. This is in agreement with the study conducted by Mahmood (2007) in fetuses of the Awassi breed of sheep. The ossification of the occipital bone occurred earlier in Yankasa than in Uda and Balami probably because of the breed and genetic difference. The densely ossifying occipital squama and the bony spicules observed in the developing interparietal part of occipital bone in this study suggest that they are endochondral (cartilages) and intra-membranous (membrane) in origin respectively, which is in line with the work of Matsumura *et al*. (1994) and Bernard *et al.* (2015) in human fetuses, who reported that the occipital bone had both membranous and cartilaginous origin. Also, according to Bernard *et al*. (2015) and Matsumura *et al.* (1994), the ossification of the human occipital squama occurred at 63rd and 84th days of gestation respectively, which were later than the 45th day reported in this work.

The zygomatic process of the frontal bone (ZPf) and frontal process of zygomatic bone (FPz) began to ossify at the 54th -56th days of gestation (2nd trimester) in the

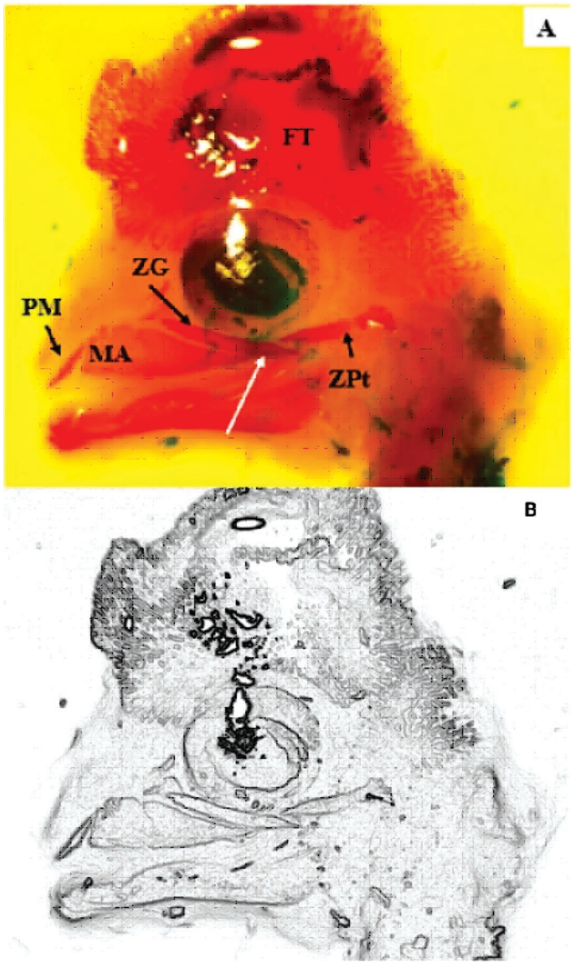

Fig. 2. A stereomicrograph (A) and bone map (B) of the lateral view of a 45th day old Yankasa fetus showing the ossifying zygomatic process of temporal bone (ZPt), the zygomatic bone (ZG), temporal process of zygomatic bone (white arrow), premaxilla bone (PM) and maxilla bone (MA), and frontal bone (FT). Alizarin red stain (5X).

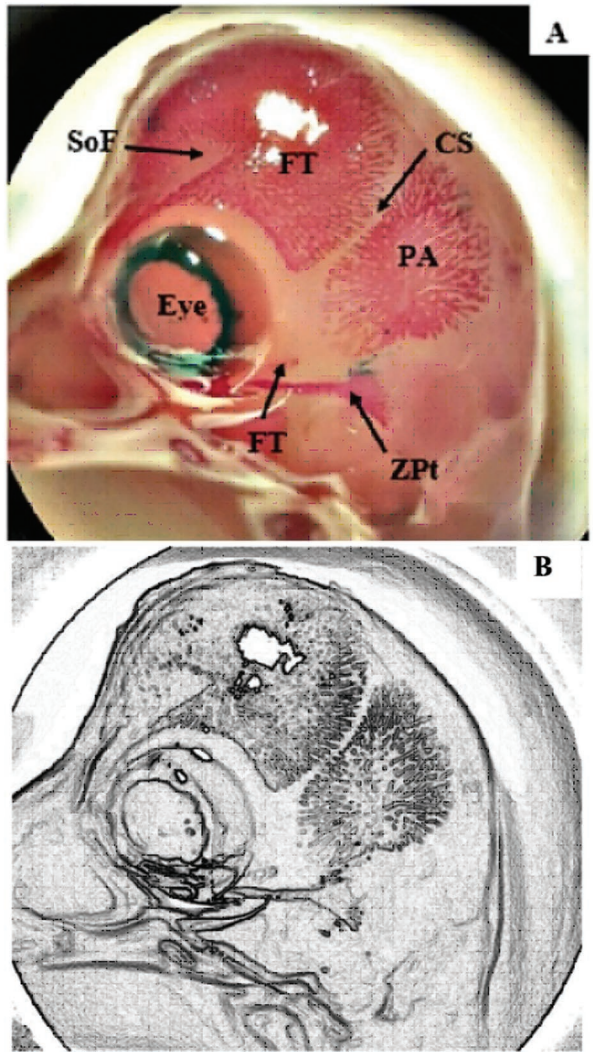

Fig. 3. A stereomicrograph (A) and bone map (B) of 48th day old Uda fetal skull, showing the developing parietal bone (PA), zygomatic process of temporal bone (ZPt), coronoid process of mandible (CrP), frontal bone (FT), supraorbital foramen (SoF), and cornual sutures (CS), Alizarin red stain (5X).

Yankasa and 61st – 67th days of gestation in Uda and Balami breeds. At these age groups, the ossifying bones were seen to have emerged as small finger-like projections growing downwards and upwards from the frontal and zygomatic bones respectively to form the cranial margin of the orbital canal, and the orbit was observed to be incomplete (Fig. 8).

The zygomatic process of the frontal bone (ZPf) and frontal process of zygomatic bone (FPz) ossified within 54th -56th days of gestation in Yankasa and 61st -67th days of gestation in Uda and Balami. The ZPf and FPz were both extensions of the frontal and zygomatic bone respectively, this justifies why they ossify at a later age compared to the frontal and zygomatic bone. The fusion of these bones determined the time of formation of the caudal border of the orbit. The time, pattern, and sequence of ossification of the ZPf and FPz were ignored by several authors that have studied the embryology of the animal skull. The temporal bone was yet to ossify across the seven age groups from day 42-67. The caudal radiation of the bony spicules from the ossifying zygomatic process of the temporal bone (ZPt) suggests that the temporal squama may share the same ossification centre with the ZPt. Hence it is possible that the squamous part of the temporal bone has a single ossification centre. However, this is contrary to the work of de Buffrénil*et al.* (2015) in reptiles where it was reported that the ossification centre

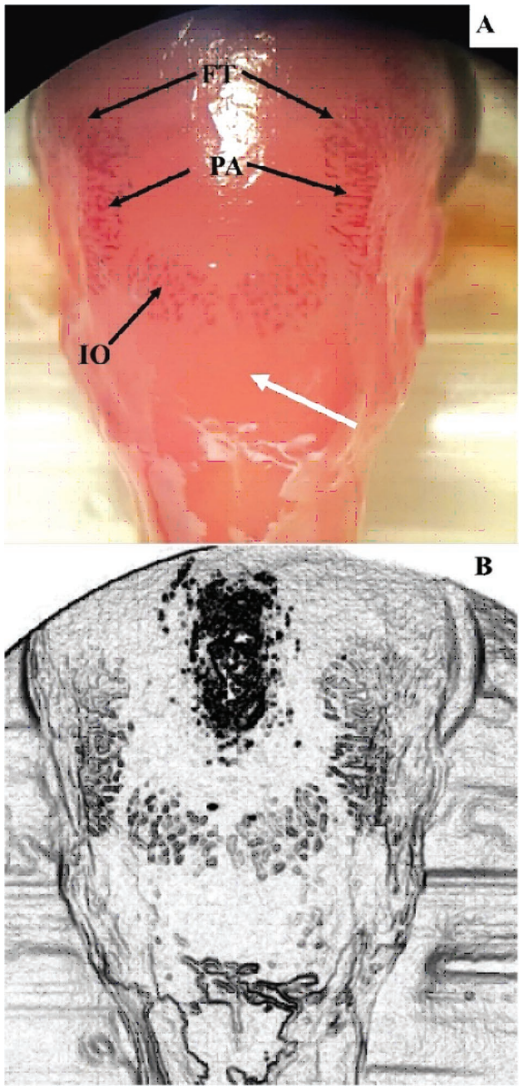

Fig. 4. A stereomicrograph (A) and bone map (B) of the caudal view of the skull in a 50th day old Uda fetus showing; the ossifying bone spicules of the frontal (FT), parietal (PA), and occipital squamous (OS), the ossification centers of the occipital and temporal bones are yet to appear (white arrow), Alizarin stain (5X).

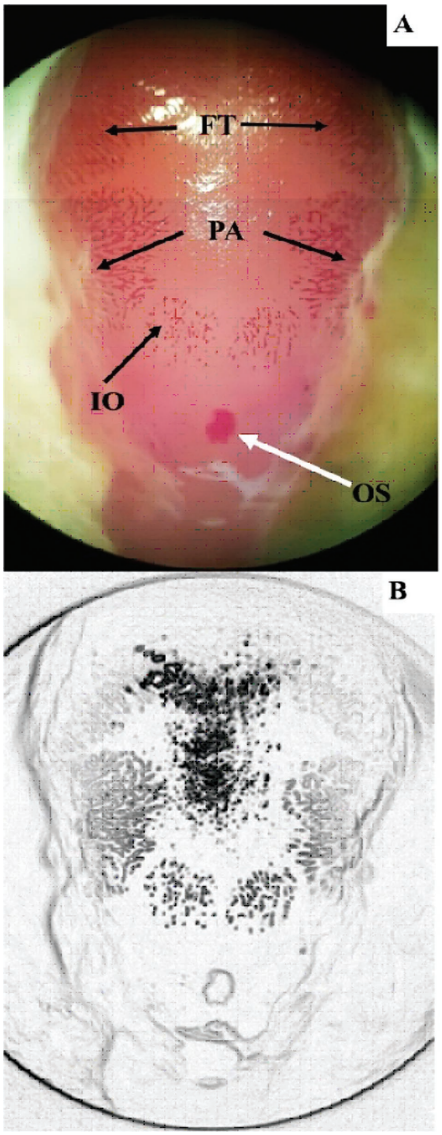

Fig. 5. A stereomicrograph (A) and bone map (B) of a 54th day old Uda fetal skull, showing a coma shaped ossification centre of the occipital squamous (OS), bone spicules of the occipital part of interparietal bone (IO), parietal bone (PA), and frontal bones (FT) ossifying towards the median plane of the skull, Alizarin red stain (5X).

for the squamosal portion of the temporal bone appeared in the region of the midpoint of the base of the squama. The ossification of the interparietal bone was yet to occur in any age group. This agrees with the findings of Martín and García-Gonzalez (2015) who reported that the interparietal bone couldn't be traced during the fetal phase in a Spanish breed of sheep (*Rasa Aragonesa*). The interparietal bone (IP) of the three breeds of sheep did not ossify throughout the seven age groups, from day 42 to day 67.

Development of the viscerocranium bones of the skull

The viscera-cranial bones of the skull; maxilla (MA), premaxilla (PM), nasal (NA), lacrimal (LC), and zygomatic (ZG) were all observed to develop independently at different stages of development. The premaxilla (PM), maxilla (MA), and zygomatic bone (ZG) were among the first of the facial bones to appear. They began ossification at 45th - 47th days of gestation across the three breeds, and at this age the PM and ZG bones appeared spindle in shape and the zygomatic bone

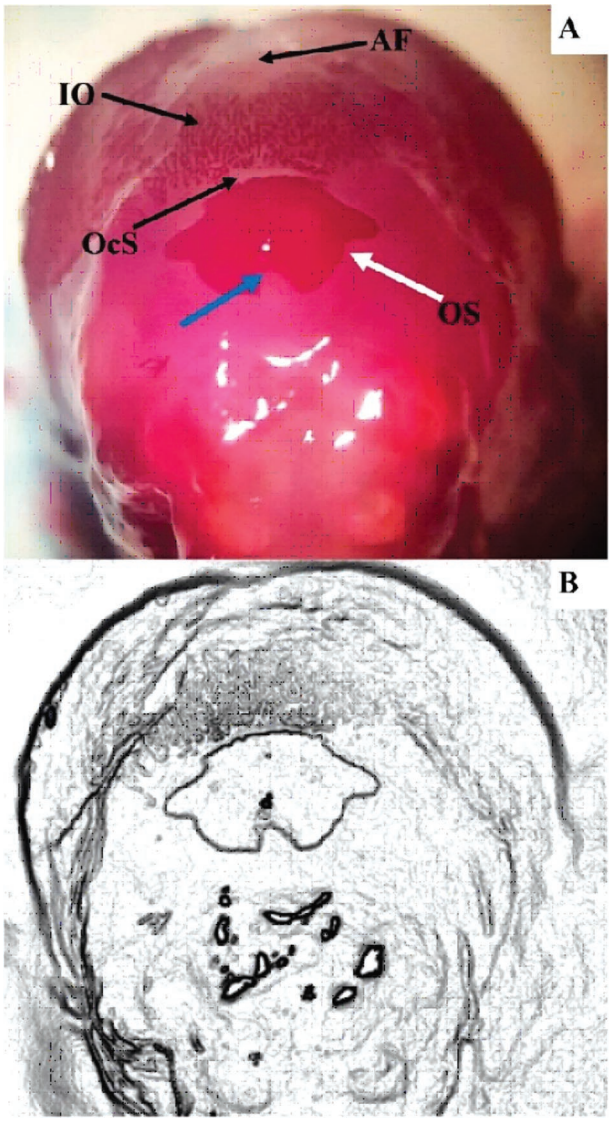

Fig. 6. A stereomicrograph (A) and bone map (B) of the caudal view of the skull of a 61st day old Balami, showing ossifying butterfly-shaped occipital squamous (OS/white arrow), occipital suture (OcS), anterior fontanelle (AF), interparietal part of occipital bone (IO) and caudal margin of the foramen magnum (blue arrow), Alizarin red stain (5X).

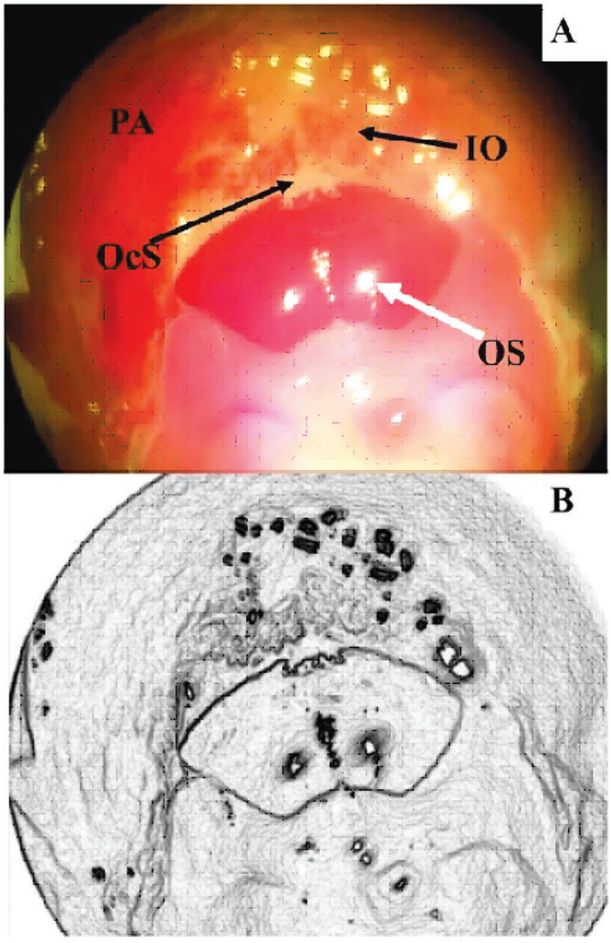

Fig. 7. A stereomicrograph (A) and bone map (B) of the caudal view of a 65th day old Balami fetus, showing developing interparietal part of occipital bone (IO), occipital suture (OcS) parietal bone (PA), arc-block shaped occipital squamous (OS/white arrow) Alizarin red stain (5X).

extended beyond the eyeball forming the temporal process of the zygomatic bone (TPz). The ossification of the maxilla bone (MA) in the three breeds of sheep began as a triangular meshwork of bone spicules between the developing premaxilla and zygomatic bone which was observed at 45th - 47th days of gestation. With the age of the fetus, the size and density of the meshwork of the bony spicules increased. The ossification of the nasal bone (NA) was observed at 54th – 56th days of gestation, it began as a meshwork of spindle-shaped bone spicules from the lateral and medial margin of each of the two nasal bones (left and right) and spread towards the centre of the bones. The development or growth of the length of the nasal bones extends rostrally from the margin of the fronto-nasal sutures to the tip of the bones (Figs.9 and10). The lacrimal bone (LC) was the last of the viscera-cranial bones to develop (at 57th – 60th days of gestation). It began as a cube-shaped meshwork of bone spicules which was later seen radiating rostrally towards the developing nasal bone at the 61st – 67th days of gestation across the three breeds of sheep (Fig.10).

In this study, no breed differences were noted in the time and sequence of ossification of the premaxilla, maxilla, zygomatic, lacrimal, and nasal bones. The premaxilla, maxilla, and zygomatic bones began to ossify within the 45th – 47th days of gestation of the 1st-trimester fetuses via intra-membranous ossification, the pattern of ossification and presence of unossified gaps between

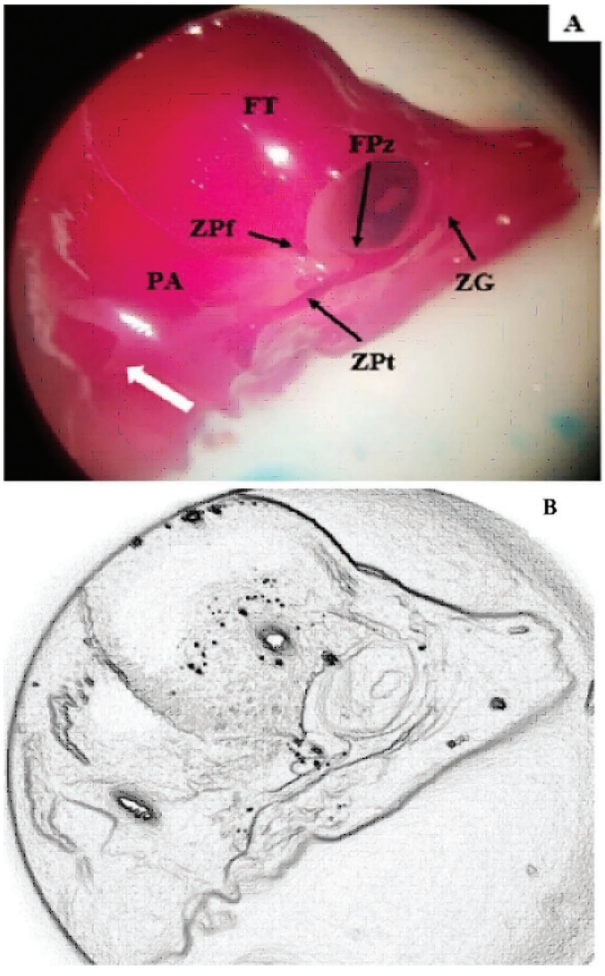

Fig. 8. A stereomicrograph (A) and bone map (B) of a 65th day old Uda fetal skull showing the ossifying zygomatic process of the frontal bone (ZPf), frontal process of zygomatic bone (FPz), occipital squamous (white arrow), frontal bone (FT), parietal bone (PA), zygomatic process of temporal bone (ZPt), and zygomatic bone (ZG), Alizarin red stain (5X).

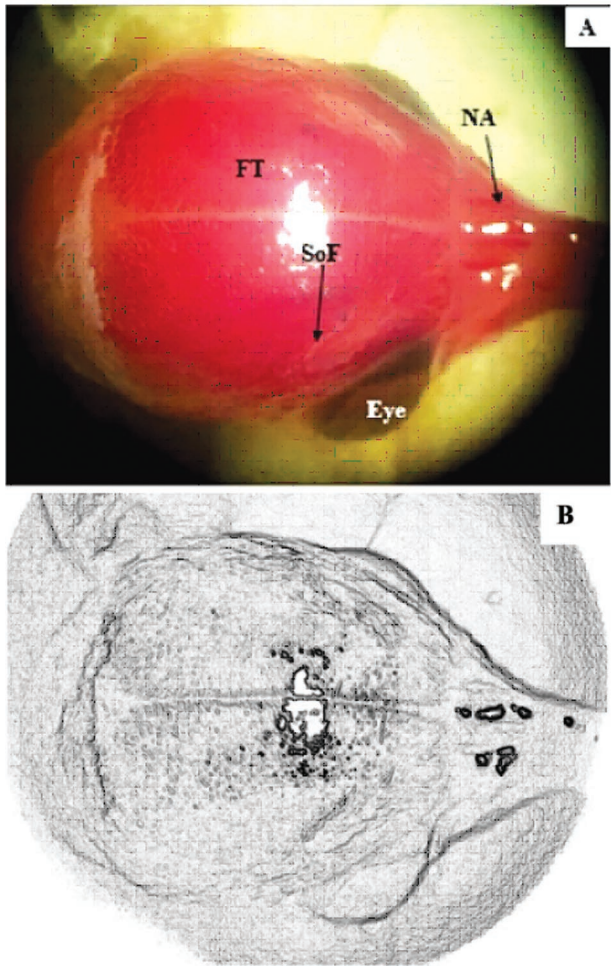

Fig. 9. A stereomicrograph (A) and bone map (B) of the dorsal view of the skull of a 55th day old Yankasa fetus showing developing left and right frontal bone (FT) with bone spicules at the merging of the frontonasal sutures, supraorbital foramen (SoF), and developing left and right nasal bone (NA) to about half its length, Alizarin red stain (5X).

these bones indicate that they all have a single and centric ossification centre. The densely ossified premaxilla and zygomatic bones observed at 45th – 47th days of gestation could suggest that they ossify earlier and grows faster than the maxilla. Details on the time of ossification of the premaxilla bone in animals is scanty, however, in humans, the ossification centre of the premaxilla appears at a little longer time, at 49th days of gestation but through an intra-membranous ossification as reported by Rice (2008). The single ossification centers of the facial bones reported in this work disagree with the study of Rice (2008) and Wood *et al.*(1969) who opined that the single ossification centre of the maxilla bone ossify and spread anteriorly to form the premaxilla. Contrary to the findings of this study and previous study, de Buffrénil *et al.* (2015) suggested that the premaxilla arises from two separate ossification centers in reptiles. The bony spicules observed in the ossification processes of the facial bones in fetuses of Indian Buffalo by Teja and Rajendranath (2017) suggest intra-membranous ossification similar to that of the present study. The earliest findings on the ossification centers of the maxilla reported that it had six ossification centers, later, several kinds of literature in human embryology has reported that the maxilla which ossified from two centers only, one for the maxilla proper and one for the premaxilla (Keibel and Mall 1910, Cunningham 1913). These centers were said to appear on the 42nd day of gestation in human fetuses and unite in the 3rd month of gestation and through intra-membranous ossification (Bernard *et al*. 2015). The result of the study

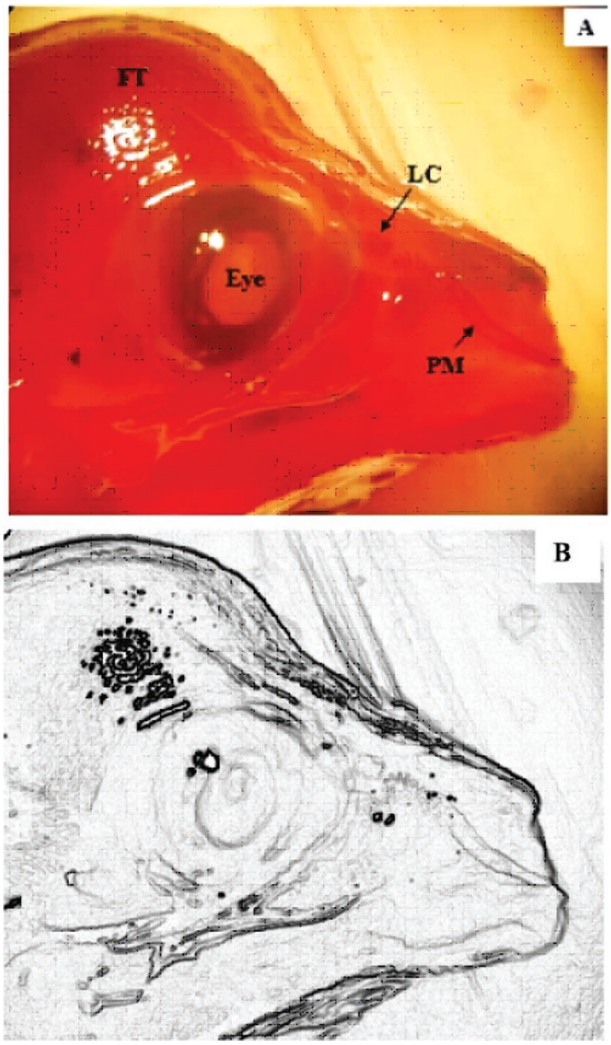

Fig. 10. A stereomicrograph (A) and bone map (B) of the cranial view of the skull of a 58th day old Balami fetus showing a cube shaped ossifying lacrimal bone (LC), frontal bone (FT), and premaxilla bone (PM) Alizarin red stain (5X).

on the Awassi sheep fetuses by Mahmood (2007) showed that the ossification centers of the maxilla and premaxilla bones appeared at 43days old (earlier than what is reported in the current study) and in zygomatic and lacrimal bones at 46 days old (similar to what we have reported), while that of nasal bone appeared at 49 days old (earlier than what we have reported). In the Awassi sheep, the ossification centers appeared in the zygomatic, lacrimal, and nasal bones approximately in the middle of each bone, the ossification spread in two directions, rostral and caudal, this location of the ossification centre and its pattern of spread is similar to the centric ossification of zygomatic and lacrimal bone reported in this study. These findings disagree with that of the nasal bone. However, the single and centric ossification centre of maxilla observed in this study disagrees with the double and eccentric ossification centers reported in Awassisheep in a study conducted by Mahmood (2007). In the Awassi sheep, the ossification centre situated at the nasal process of premaxilla disagree with the centric ossification centre we observed in the premaxilla of Nigerian breeds of sheep and the pattern of ossification also varies (Mahmood 2007). These variations in the ossification could be due to breed and environmental differences as suggested by Workalemahu (2018) and Sacks (2004) who stated that complex interactions between genetic and environmental factors such as maternal nutrition and placental function may play important roles in fetal growth.

CONCLUSION

The neurocranial and viscerocranial bones developed via intra-membranous ossification except the occipital that had an additional endochondral origin. Generally, the bones of the skull appeared and developed faster in Yankasa than Balami and Uda. The alizarin findings showed that the ossification centers of all the bones of the neurocranium were eccentric in origin, while that of the viscero-cranium were centric except the nasal bones which had eccentric ossification centers. All paired bones had paired ossification centers and all single bones showed a single ossification centre.

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